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Topic: Development and evaluation of novel topical formulation(s) for enhancing combat efficiency of troops

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Introduction:

Excessive bleeding is the primary factor of avoidable death in both civilian trauma centers as well as military battle field. It is well known that huge loss of blood reduces the blood volume for circulation, leading to obstruction of human body microcirculation and hypoxia in brain and other organs. Furthermore, such casualties are more vulnerable to coagulopathy, acidosis and hypothermia, which lead to increased risk of morbidity and mortality from sepsis and multiple organ failure.

Hemostasis is a physiological process that stops bleeding at the site of an injury while maintaining normal blood flow elsewhere in the circulation. Blood loss is stopped by formation of a hemostatic plug. The endothelium in blood vessels maintains an anticoagulant surface that serves to maintain blood in its fluid state, but if the blood vessel is damaged, components of the sub-endothelial matrix are exposed to the blood. Several of these components activate the two main processes of hemostasis to initiate formation of a blood clot, composed primarily of platelets and fibrin. This process is tightly regulated such that it is activated within seconds of an injury but must remain localized to the site of injury. There are two main components of hemostasis. Primary hemostasis refers to platelet aggregation and platelet plug formation. Platelets are activated in a multifaceted process, and as a result they adhere to the site of injury and to each other, plugging the injury. Secondary hemostasis refers to the deposition of insoluble fibrin, which is generated by the proteolytic coagulation cascade. This insoluble fibrin forms a mesh that is incorporated into and around the platelet plug. This mesh serves to strengthen and stabilize the blood clot. These two processes happen simultaneously and are associated with each other (Gale, 2011).

Hemostatic agents improve hemostasis by multiple mechanisms that include improving primary hemostasis, stimulating thrombin generation and/or fibrin formation or inhibiting fibrinolysis.

Hundreds of Indian troops die every year due to blood loss caused by combat related injuries. During Kargil war and most of the recent naxalite operations, most of such casualties have been due to gunshot wounds, shrapnel and splinter injuries. These deaths are avoidable. These deaths can be prevented to a large extent by making available a suitable hemostatic agent in emergency medical kit of our Armed Forces and Central Armed Police Force (CAPF). There are many

hemostatic agents that are commercially available but their practical applicability in battlefield conditions during combat operations is limited due to their exorbitant cost, non- availability in desired quantities, side-effects like thermal injury and burns resulting from the exothermic reaction after application, poor biodegradability and difficulty in removal of certain hemostatic agents at the time of surgery . In view of existing technology gap, there is a need to develop an indigenized, efficacious and cost-effective hemostatic agent, preferably from easily available materials for the use of our defence force personnel during combat operations on the battlefield/ during their evacuation to proper hospital set-up as a life-saving measure.

On the basis of literature survey done and preliminary screening experiments done at INMAS, the present study aims to evaluate a combination of Gum Tragacanth with calcium gluconate as a potential hemostatic agent, which may be more effective, less expensive and readily available as compared to the existing options. The use of gauze dressings, sponges, and direct pressure has long been adopted as a method of choice to stop bleeding in pre-hospital care. So the present work involves development of gum Tragacanth based hemostatic topical formulations, which will not possess any cytotoxicity, sub-acute toxicity effects, genetic toxicity, hemolysis, and shall have good biological compatibility and biodegradable properties.

Gum Tragacanth is a natural gum obtained from the dried sap of several species of Middle Eastern legumes of the genus *Astragalus*, including *A. adscendens, A. gummifer, A. brachycalyx* and *A. tragacantha*. It is a viscous, odorless, tasteless, water-soluble mixture of polysaccharides acquired from sap that is drained from the root of the plant and dried. The gum seeps from the plant in twisted ribbons or flakes that can be powdered. It absorbs water to become a gel, which can be stirred into a paste. The major fractions are known as tragacanthin and bassorin. Tragacanthin is highly water soluble as a mucilaginous colloid and bassorin is far less soluble but swells in water to form a gel.

The gum has traditionally been used as a herbal remedy for conditions like cough and diarrhea. As a mucilage or paste, it has been used as a topical treatment for burns. It is also used in pharmaceuticals and foods as an emulsifier, thickener, stabilizer, and texturant additive (E number E413). It is traditionally used as a binder, as it does not adhere to itself the same way other gums (such as gum arabic) do when dry. Its water solubility is ideal for ease of working and an even spread, and it is one of the stronger gums for holding particles in suspension.

In addition with the gum the study also emphasize on the role of calcium in the blood clotting process. Calcium ions are well known to accelerate the formation of fibrin clot from fibrinogen in the presence of thrombin. The conversion of fibrinogen to fibrin clot is a multistep process, and many studies have attempted to examine the specific step influenced by calcium (Brass, 2019).

Therefore the current study focuses to develop hemostatic dressing material(s) incorporated with the Gum Tragacanth and Calcium Gluconate with enhanced efficacy and safety with lower cost and easy availability compared to other hemostatic materials existing commercially.

Literature Review

Nugraha and Suwarso in 2018, reported the in vitro hemostatic activity of ethanol extract of beetroot (Beta vulgaris L), which was performed on whole blood of rat by Lee-White method to determine the clotting time and the Eustrek method to observe microscopic picture of clotted blood. The study exhibited that hemostatic activity of ethanol extract of beetroot showed a dose-dependent manner, i.e. at the concentration of 1% and 2% decreased the clotting time to 24.4+1.14 and 15.9 + 0.65, respectively, as compared to EDTA treatment which did not clot for 120 min ($p<0.05$) and microscopically showed that blood cells appeared to be attached to each other at the concentration of 1% and 2% of ethanol extract of Beetroot. (Nugraha & Suwarso, n.d.).

Jae-Young Byun *et al.*, in 2018 demonstrated the hemostatic efficacy of ferric subsulfate and chitosan-based styptics as a powder and a gel containing ferric subsulfate & chitosan and a soaked pad containing ferric sulfate & lidocaine with the help of rat tail bleeding model, in which rat tail tips were transacted, after which styptics were applied with pressure and wounds were observed for hemostasis for 3 min, then irrigated with saline to check for recurrent haemorrhage. They also evaluated the cytotoxicity of these compounds using cell counting kit-8 assay against L292 mouse fibroblast. (Byun, Lee, Lee, & Yoon, 2018)

WO 2017/060821 Al disclosed a hemostatic composition comprising of locust bean gum / xanthan gum / guar gum with calcium gluconate which claimed to increase blood stopping efficiency, and providing the possibility of transporting the wounded after formation of artificial thrombus. This claim was successfully revealed by formation of artificial thrombus with optimal moisture and reduced adhesive properties for preventing further injury of wound edges (Bureau, 2009).

Xiao Yang *et al.*, in 2017, provided a review on polysaccharide-based hemostatic materials and agents, including their advantages and drawbacks in the hemostatic application. Polysaccharides are naturally derived polymers having excellent biodegradable and biocompatible properties.

Furthermore, along with hemostatic material, they also incorporated anti-microbial agents into the test material (Yang et al., 2017).

Lt Col Vivek Kumar Singh in 2017conducted an observational study to analyze tactical combat casualty care of 707 Indian Army casualties admitted in a tertiary care hospital and assess the survival outcomes. The study included the type of casualties, their mode of evacuation, patient survival and outcome. This study concludes that, due to absence of night flying, two casualties were forced to be evacuated by road. One casualty expired during evacuation due to gunshot wound of chest and exsanguinations. Patients who required surgery were operated upon within five hours, thus achieving maximum survival benefit.

Georg Kaehler *et al.*, in 2015 conducted a randomized prospective comparative study regarding the potential of an endoscopically applicable polysaccharide powder (Endoclot) in pigs receiving antithrombotic medication. At the end of the study, endoscopic hemostasis was successful in all cases and post-interventional bleeding was not recorded. Furthermore they concluded that the Polysaccharide powder was able to stop active bleeding from Forrest I b lesions in pigs receiving antithrombotic therapy (Kaehler, Dutenhoefner, & Magdeburg, 2015).

Monica Bhatnagar et al., in 2013 evaluated the potential of polymeric component of aqueous extract of gum acacia and the seeds of M. oleifera in wound management. Results of their study revealed that both biopolymers were hemostatic and accelerated blood coagulation without any toxicity. They were biodegradable and exhibited good water absorption capacity. (Bhatnagar, Parwani, Sharma, Ganguli, & Bhatnagar, 2013).

JP Garner and RFR Brown in 2012 reviwed the latest advances in haemostatic technology and evaluates those agents that have a potential utility in traumatic hemorrhage control. Particular attention is paid to those agents that might be easily incorporated into a field dressing or bandage for prehospital use rather than hemorrhage control at surgery(Garner & Brown, 2008).

P. V. Mohanan et al., in 2011 disclosed the hemostatic efficiency of poly [*ß*-(1, 4)-2-amino-2-deoxy-Dglucosamine]- based hemostatic dressing material on albino rabbits. They characterised the dressing material with SEM which revealed that the chitosan dressing was structured in a 3D macroporous network that offered good accessibility to the wound and better blood absorption.

Furthermore in vitro tissue culture study on L-929 cells and skin irritation study were also carried out for the assessment of cell cytotoxicity and non-skin irritant property of the dressing material, which confirmed that the material did not induce any cytotoxicity and skin irritation after the application of the dressing material. (Mohanan, Mavely, & Pandya, 2011).

There is another review presented by **Tao Peng in 2010** about broad spectrum of hemostatic agents and new technologies related to the modification and fabrication of hemostatic agents to develop better physical structure for hemorrhage control, according to this review only self-expanding polymer is the only synthetic polymer with potentials for uncontrolled hemorrhage, rest of all hemostatic agents have some disadvantage in managing excessive bleeding. This review also concludes the necessity of manual pressure to keep the solid biomaterials at a bleeding site when using solid biomaterials(Peng, 2010).

Rationale of study

Excessive blood loss due to injuries sustained on the battlefield is the common crisis associated with the death of military and paramilitary personnel. Haemostasis is therefore an essential and critical procedure to minimize blood loss, and to reduce the risk of death caused by hemorrhage. Therefore, a suitable, efficacious and cost-effective hemostatic material needs to be developed to minimise these preventable deaths on the battlefield. On the basis of literature survey done there are several hemostatic materials, formulations and devices commercially available, which are based on biopolymers, synthetic polymers, bio-synthetic polymers, ceramics and polymer-ceramic composites and. However, most of them suffer from one or the other limitation, restricting their use in the Indian military and paramilitary forces.

In addition there is pool of researchers who are working to improve the efficiency of existing Hemostatic agents as well as developing new hemostatic agents having faster coagulation time, better absorbency etc.

Besides above-mentioned agents, some studies have also shown the applicability of natural gums as potential hemostatic agents (Bhatnagar et al., 2013; Bureau, 2009). Based on the available literature it is envisaged that natural gums because of their high absorbing capacity, neutral pH, less systemic absorption, high therapeutic index may offer an excellent hemostatic agent/s.

Therefore the present work has been envisaged to evaluate the potential of Natural gums as hemostatic agents, followed by development of formulations/devises comprising natural gums.

Aim and objective

Aim: Development and evaluation of novel topical formulation(s) for enhancing combat efficiency of troops.

Objectives:

1. Evaluation of Natural Gums like Gum Tragacanth, Guar Gum, Xanthan Gum etc. as potential hemostatic agents
2. Development and evaluation of absorbable dressing materials impregnated with Natural Gum/s along with pharmaceutical excipients.
3. In vitro and in-vivo evaluation of the hemostatic dressing materials for its efficacy and safety.

Plan of work

1. Evaluation of Natural Gums like Gum Tragacanth, Guar Gum, Xanthan Gum etc. as potential hemostatic agents

In-vitro Studies:

A. Blood coagulation study by Whole blood clotting time (Lee and White Method):

WBCT will be measured using a Lee and White method.7mg of Test material will be added into three potassium EDTA (10%) coated glass tubes with 2 mg calcium gluconate monohydrate and dissolve it in 100µl distilled water. In each tubes about 1ml of Fresh blood will be collected from retro orbital plexus of healthy Sprague Dawley rats. The tubes will be incubated at 37°C in a water bath for 5 min prior to the observation of blood coagulation. At the time of adding blood into each tubes, the stopwatch will be running to determine the clotting time. At the interval of 30 secs, each tube will be tilted and the blood clotting will be observed until coagulation. The WBCT will justify as the time from the start of the blood collection until the end of the blood coagulation in all tubes. The experiment will be repeatedly performing five times for each test sample.

B. Microscopic examination by Eustrek Method:

6 pieces of object glass will be prepared (Normal blood, EDTA treats blood and 2 for each test material). One drop of blood samples will be taken from healthy Sprague Dawley rat and put it on object glass and spread it with the help of another object glass. The blood clot will be observed under microscope (40X).

C. Plasma recalcification time:

PRT will be measured using a hook method. 90 mL of human whole blood taken from a healthy volunteer and mix with 12.6 mL of anticoagulant citrate phosphate dextrose adenine solution (41.2 mg of citric acid (hydrous), 0.332 g of sodium citrate (hydrous), 28 mg of monobasic sodium phosphate (hydrous), 0.366 g of dextrose (anhydrous), and 3.46 mg of adenine).

To separate blood corpuscles and obtain PPP, the anticoagulated blood will be centrifuged at 5135 X *g* for 12 min at 4°C. 300 µl of PPP will be taken and add into a glass test tube that primarily contained 0.003 g of a given testing material. The whole

tube will be allowed for incubation at 37°C for 1 min. The plasma will be then recalcified by adding 30µL of 0.5*M* CaCl2 solution. Fibrin formation in the plasma solution will be observed by hooking the plasma solution every 30 s. The PRT will be counted from the start of the addition of the $CaCl_2$ solution until the first sign of the fibrin formation on a hook. The experiment will be repeated 4 times per each testing material.

D. Platelet adhesion and activation studies:

The 3.2% sodium citrate solution as an anticoagulant will be added to whole blood and centrifuged at 100×*g* for 10 min to obtain platelet-rich plasma (PRP). The PRP will be immediately processed within 2 h after collection. Test material will be washed three times with deionized water and subsequently rinsed with 0.1*M* phosphate buffer (PB). The sample will be promptly introduced into the wells of 24-well tissue culture polystyrene plates and fixed to the bottom of the wells with *o*-rings. PRP (650 µL) will be added into the surfaces of each sample and incubated for 10 or 60 min at 37°C. The samples will be washed again with 0.1*M PB* three times to remove any non-adhered platelets and plasma proteins. The adhered platelets are fixed with 2% glutaraldehyde solution in 0.1*M PB* for 1 h. The samples will be later washed three times with 0.1*M* PB, dehydrated by ethanol graded series, and dried using a critical point CO2 method. Finally, the samples will be coated in vacuum with gold. The platelets on each sample will be examined by scanning electron microscopy. The samples will be tested in duplicate.

E. Platelet aggregation.

One millilitre of stirred citrated PRP will be allowed to contact with 3.5 mg of each hemostatic agent pre moistened with 100 µl of phosphate-buffered saline (PBS) in a siliconized glass cuvette. Aliquots of supernatant (100 µl) will be removed every minute for 5 min and the platelet count will be measured in triplicate utilizing an electronic cell counter. Platelet count from each experimental aliquot will be normalized using count from unreacted PRP. For each hemostatic agent three independent sets of experiment will be performed using the hemostatic sponge agent pre moistened with 1 unit of thrombin and PBS. These experiments will also be performed three times. Platelet aggregation from 1 unit of thrombin alone will be used as a positive control.

F. In vitro cytotoxicity study

In vitro cytotoxicity studies of testing material based hemostatic dressing samples will be carried out with the L929 (mouse fibroblast subcutaneous connective tissue) cell line. The cells will be maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, pencillin (100 IU/ml), and streptomycin (100 mg/ml).The culture will be incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide (24–26 h) with a medium change at an interval of 3 days. Phenol as negative and high density polyethylene (USP grade) will be used as negative controls for the study. The culture medium and the specimen will be removed and the cell cytotoxicity will be evaluated qualitatively. The qualitative evaluation of cytotoxicity involves examining the cells microscopically to assess for changes in general morphology, vacuolization, detachment, cell lysis, and membrane. The changes from normal cell morphology will be recorded as none, slight, moderate, and severe, depending upon the extent of cell damage.

In-vivo Study :

A. Evaluation of haemostatic potential

After general anaesthesia with a mixture of ketamine and xylazine, six healthy, adult rabbits (2000–3000 g) will be shaved and disinfected on both ears. Using a longitudinal incision, 1.0 cm wound segments will be made on the right and left marginal ear arteries. Through the resultant jet spray of blood, the right wound will be immediately covered with 2 × 2 cm2 piece of testing material based dressing of known weight (w1).

Similarly, the left wound (1 cm) will be coverd with Normal Hemostatic bandage (control) of known weight (w2). Direct pressure will be applied for 2 min and then the samples will be removed and weighed immediately (w3 for test and w4 for control) after hemostasis. All the blood that oozed out from the initial cut on the arterial surfaces will be absorbed by both types of materials (test and control). Hemostasis is complete when bleeding will be no longer observed in the wound site. The time taken for hemostasis will be noted. Blood loss (w3–w1 for the test and w4–w2 for control) will be calculated from the materials weighted before and after absorbing blood. The following parameters will be evaluated from the study.

B. Hemostatic Effects in a Rat Model with Severe Femoral Artery Bleeding:

Anesthesia will be induced with Ketamine and the right leg will be shaved and femoral artery will be pierced with a 24-gauge needle, and active severe arterial bleeding will be achieved. The wound will be immediately covered with a gauze pad to remove excess blood. After the excess blood had been blotted, 0.25 g testing material will be poured directly into the bleeding site, and a 200-g scale weight will be placed on it for 30 sec so that a standard level of compression could be achieved. At 30-sec intervals, the bleeding from the site will be evaluated by an observer with a stopwatch who will report whether or not it will stop. After 30 sec, the scale weight will be removed and hemostasis will be assessed. If the bleeding had stopped, the test will be scored as "passed at 30 sec." If the bleeding did not stop, the same quantity of testing material and the same amount of compression will reapply for an additional 30 sec in a similar way. If bleeding stopped after the second application, the test will be scored as "passed at 60 sec." If bleeding continued after the second application, a third and final time trial of the same procedure will be repeated for an additional 30 sec. If the bleeding stopped after this trial, the test will be scored as "passed at 90 sec." If hemostasis would not be able to achieve after the third application, the test will be scored as "failed," and bleeding will stop by means of additional compression, with ligation of the vessel performed as needed.

C. Skin irritation study

Healthy, smooth skinned rabbits with a body weight range between 2000–3000 g will be used for the study. The fur on the back of the animal (dorsal side) will be clipped closely and the skin will be ensured to be free of mechanical trauma or signs of irritation and disinfected with 70% alcohol. The test material will be moistened slightly with physiological saline and applied topically on the upper left side of the animal. Similarly, a sterile gauze piece (6 cm2) moistened with physiological saline will be applied on the lower left side of the animal. Application sites will be covered with an occlusive bandage for 4h. All the applied sites will be observed at 1, 24, 48, and 72 h after removal of patches for the evidence of any tissue reactions. Grading of tissue reaction for erythema and edema will be done and the primary irritation index will be calculated by adding the scores of each animal and dividing the total by the number of animals.

2. Development and evaluation of absorbable dressing materials impregnated with Natural Gum/s along with pharmaceutical excipients

- Selection of absorbable dressing material
- Optimistation and evaluation of Impregnated absorbable dressing Material comprising Natural Gum/s.
- In vitro characterization of developed dressing material

3. In vitro and in-vivo evaluation of the hemostatic dressing materials for its efficacy and safety.

- Cytotoxicity
- Skin Irritation
- Skin Sensitization
- Acute Intravenous Systemic Toxicity
- Acute Intraperitoneal Systemic Toxicity
- Hemolysis Test
- Pyrogenicity Test

- **Heavy metal testing**

The subject device was tested for cadmium, lead, arsenic and mercury. The levels for all elements were found to be fewer than 1 ppm, which meets the USP-232 limits.

- **Bench performance testing**

- Physical Properties
- Absorbency Testing
- Moisture Content
- pH Testing
- Tensile strength
- In-Vitro Hemostatic Activity
- In Vivo rabbit hemostasis study

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